

# Protein C activation by an activator purified from the venom of *Agkistrodon halys halys*

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# Protein C activation by an activator purified from the venom of *Agkistrodon halys halys*

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The protein C activator from *Agkistrodon halys halys* venom was purified 533-fold by ion-exchange chromatography on QAE-Sephadex A-50, affinity chromatography on aprotinin-Sepharose and Mono-Q fast protein liquid chromatography. The purified enzyme is a single chain protein with an apparent molecular weight of 36 000 that activates protein C by proteolytic removal of a small fragment from the heavy chain. The protein C activator exhibited a high amidolytic activity towards the tripeptide substrates D-Pro-Phe-Arg-pNA (S2302) and D-Phe-(piperidyl)-Arg-pNA (S2238). The activity of the activator was not affected by thiolprotease or metalloprotease inhibitors. The activator was inhibited, however, by benzamidine, Phe-Pro-Arg chloromethyl ketone, *p*-nitrophenyl *p*-guanidinobenzoate and soy bean trypsin inhibitor, which classifies the enzyme as a serine protease. The purified protease was capable of activating both human and bovine protein C. Activation of human protein C only occurred at an appreciable rate in a calcium-free reaction medium at low ionic strength.  $\text{Ca}^{2+}$  ions inhibited the activation of human protein C with an apparent  $K_i$  of 0.8 mM. Addition of NaCl to the reaction medium also strongly inhibited human protein C activation (50% inhibition at 20 mM NaCl). Kinetic analysis of human protein C activation by the venom activator (in a calcium-free medium) revealed an apparent  $K_m$  for protein C of 0.52  $\mu\text{M}$  and a  $k_{\text{cat}}$  of 0.17  $\text{s}^{-1}$  at  $I = 0.05$  ( $k_{\text{cat}}/K_m = 3.3 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$ ). At  $I = 0.15$  rates of human protein C activation became linear with protein C indicating a strong increase in  $K_m$  with increasing ionic strength. Activation of bovine protein C was hardly affected by variation of  $\text{Ca}^{2+}$  and NaCl concentrations in the reaction medium. The apparent  $K_s$  for calcium ion and NaCl inhibition of bovine protein C activation were  $> 10$  mM and 220 mM, respectively. At  $I = 0.1$  and in the absence of  $\text{Ca}^{2+}$  ions bovine protein C was activated with a  $K_m$  of 0.056  $\mu\text{M}$  and a  $k_{\text{cat}}$  of 0.24  $\text{s}^{-1}$  ( $k_{\text{cat}}/K_m = 4.3 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$ ). Our data are indicative for a rather large conformational and/or structural difference between human and bovine protein C at physiological ionic strength.

**Key words:** Human and bovine protein C activation, *Agkistrodon halys halys* venom, snake venom activator.

## Introduction

Protein C is a vitamin K-dependent glycoprotein that circulates in the blood as the zymogen of the serine protease, activated protein C.<sup>1</sup> Protein C ( $M_r = 62\,000$ ) consists of a heavy chain ( $M_r = 41\,000$ ) and a light chain ( $M_r = 21\,000$ ) linked via a disulphide bridge.<sup>2</sup> After activation protein C is converted into activated protein C (APC), which is an efficient inhibitor of blood coagulation whose anticoagulant properties have been attributed to its ability to inactivate factor  $\text{V}^{3,4}$  and factor  $\text{VIII}^{5-7}$  and to promote fibrinolysis.<sup>8</sup> *In vivo* protein

C activation occurs at the endothelial cell surface and is catalysed by the thrombin-thrombomodulin complex<sup>9</sup> which in case of human protein C removes a dodecapeptide from the amino terminal end of the heavy chain.<sup>2</sup>

Protein C is also activated by trypsin,<sup>10</sup> the factor X activator from Russell's viper venom<sup>10</sup> and by proteases from the venoms of the Southern copperhead, *Agkistrodon contortrix contortrix*<sup>11-16</sup> and the tropical moccasin, *Agkistrodon bilineatus*.<sup>17</sup> Finally, an acti-

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the protein C antigen concentration by specific ELISA and using a M<sub>r</sub> 56 000.

The protein C activator present in crude *Agkistrodon halys halys* venom was purified as follows. 722 mg crude venom dissolved in 35 ml 25 mM Tris/HCl (pH 7.5 at 4°C), 50 mM NaCl, 1 mM EDTA was applied to a QAE-Sephadex column (2.5 × 21 cm) equilibrated in the same buffer and the column was washed with three column volumes 25 mM Tris/HCl (pH 7.5 at 4°C), 100 mM NaCl, 1 mM EDTA. Venom proteins that adhered to the column were eluted with a linear salt gradient of 2 × 250 ml from 100 to 500 mM NaCl. Column fractions were tested for the presence of a protein C activator as described below. The activator adhered to the column and eluted in the gradient at about 200 mM NaCl. Fractions containing the protein C activator were pooled and dialysed against 20 mM hydroxyethylpiperazine ethanesulphonic acid (HEPES) (pH 7.5) and subsequently applied to an aprotinin-Sepharose column (35 mg aprotinin coupled to 10 ml CNBr-Sepharose according to the manufacturer's instructions). The protein C activator bound to the aprotinin-Sepharose column which was then washed with five column volumes 20 mM HEPES (pH 7.5), 50 mM NaCl and developed with a linear salt gradient (2 × 40 ml) of 50–300 mM NaCl in the same buffer. The activator eluted at about 125 mM NaCl. The fractions containing protein C activator were pooled, dialysed against 25 mM Tris (pH 7.5 at room temperature), 50 mM NaCl, 1 mM EDTA and subjected to ion-exchange chromatography on a Mono-Q column connected to an FPLC-system of Pharmacia. The activator adhered to the column and was eluted with a linear salt gradient of 50–300 mM NaCl. To remove the final contaminants the fractions containing activator were pooled, dialysed against 25 mM Tris (pH 7.5 at room temperature), 50 mM NaCl, 1 mM EDTA and rechromatographed on Mono-Q. The column was washed with 10 column volumes start buffer and developed with a linear gradient of 50–150 mM NaCl (5 ml total volume). Elution with 150 mM NaCl containing buffer was continued during which the activator eluted as a single peak.

#### Protein C activation

During the purification of the *Agkistrodon halys halys* activator protein C activation was routinely determined as follows. 40 µl column fraction, appropriately diluted in 20 mM HEPES (pH 7.5), 1 mM EDTA, 0.5 mg/ml ovalbumin, was preincubated at 37°C for 5 min and protein C activation was started by adding 10 µl of 1 µM bovine protein C (prewarmed) in the same buffer. After an additional 5 min a 25 µl aliquot of the activation mixture was transferred to a cuvette with

475 µl of a buffer containing 50 mM Tris (pH 7.9 at room temperature), 175 mM NaCl, 20 mM EDTA, 0.5 mg/ml ovalbumin and 192 µM S2366. From the  $\Delta A_{405-500 \text{ nm}}$  determined on a dual wavelength spectrophotometer the amount of activated protein C was calculated using the kinetic parameters for S2366 conversion by bovine APC given by the manufacturer.

Initial rates of protein C activation by the purified activator were determined in a total reaction volume of 250 µl as follows. Varying amounts of protein C were preincubated for 5 min at 37°C in a reaction buffer (composition given in the legends to the figures) and protein C activation was started by the addition of 10 µl of purified activator in the same buffer. Aliquots of 25 µl were removed at 1, 3 and 5 min from the reaction mixture and assayed for activated protein C with S2366 as described above. Quantitation of human APC was based on the kinetic parameters of S2366 conversion by human APC reported by Sala *et al.*<sup>24</sup> Rates of protein C activation were linear with time and proportional to the amount of activator added and expressed in nM APC formed per min. Further experimental details are given in the legends to the figures.

#### Gel electrophoresis

Electrophoresis of proteins was carried out as described by Laemmli<sup>25</sup> on 10% polyacrylamide gels (6% stacking gel) in the presence of SDS. After electrophoresis the gels were stained for protein with Coomassie Brilliant Blue R250. Zymographic detection of the protein C activator was carried out using the amidoblot procedure described earlier.<sup>26</sup> To this end the activator was transblotted on to nitrocellulose and SDS was removed by soaking the nitrocellulose sheet for 3 h at room temperature in 25 mM Tris (pH 7.9), 90 mM NaCl containing 2.5% Triton X-100 followed by four washes (7.5 min) in 25 mM Tris (pH 7.9). The nitrocellulose sheet was subsequently placed on an agarose gel of 1.25% agarose in 25 mM Tris (pH 7.9), 1 mM EDTA, 480 µM S2366 and 0.5 µM bovine protein C. If there is protein C activator present on the nitrocellulose sheet APC is formed which liberates *p*-nitroaniline from the chromogenic substrate in the agarose and generates a yellow band on the nitrocellulose sheet. This band can be photographed by transillumination with UV-A light to achieve optimal contrast.

## Results

#### Purification of the protein C activator from *Agkistrodon halys halys* venom

The protein C activator was purified from the crude venom by a combination of standard chromatographic techniques involving ion-exchange chromatography

**Table 1.** Purification of a protein C activator from the venom of *Agkistrodon halys halys*

	Protein <sup>a</sup> (mg)	Specific activity nmole APC/min/mg	Yield (%)	Purifi- cation (-fold)
Crude venom	722	0.6	100	1
QAE-Sephadex	41	3.5	33	5.8
Aprotinin- Sephadex	11.4	12.0	31	20
Mono-Q 1	0.9	98	20	163
Mono-Q 2	0.17	320	13	533

<sup>a</sup> Protein concentrations were calculated from the absorbance at 280 nm assuming  $A_{280}^{1\%} = 10$ . Further details are described under Materials and methods and in the text.

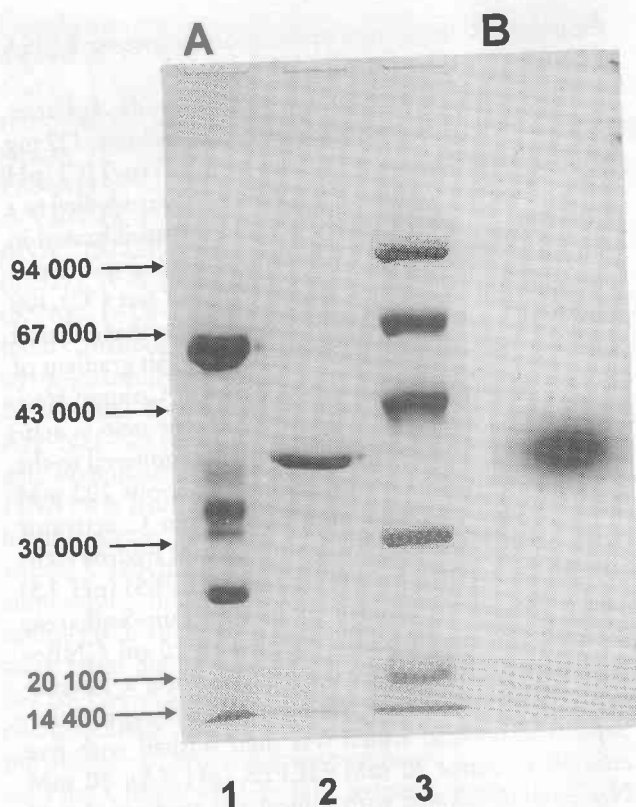
on QAE-Sephadex, affinity chromatography on aprotinin-Sephadex and repeated ion-exchange chromatography on a Mono-Q column connected to a Pharmacia FPLC system. Table 1 summarizes the purification procedure (details are given under Materials and methods) which resulted in 533-fold purification at a 13% overall yield.

Figure 1A shows a non-reduced SDS-polyacrylamide gel of the crude venom (lane 1), the purified activator (lane 2) and protein standards (lane 3). The purified activator appeared homogeneous and pure (> 95%) and migrated as a single band with an estimated  $M_r$  of 36 000. SDS-PAGE of reduced samples also showed a single band at 36 000  $M_r$ , indicating that the activator is a single chain protein (not shown).

The protein C activator on the gel was also identified by a modification of the amidoblot procedure described by Tans *et al.*<sup>26</sup> The purified protein C activator was subjected to SDS-PAGE and transblotted on to nitrocellulose. The nitrocellulose sheet was washed and subsequently placed on a layer of agarose containing protein C and the protein C-specific chromogenic substrate S2366 (Figure 1B). The appearance of a yellow band (i.e. paranitroaniline production from S2366 by APC generated in the agarose) at the migrating distance of the protein band (lane 2 Figure 1A) indicates that the protein visible on the Coomassie blue stained gel and the protein C activator are one and the same protein. In this respect it should be mentioned that the appearance of the yellow band was not due to direct conversion of S2366 by the purified venom protein since omission of protein C from the agarose resulted in a complete loss of *p*-nitroaniline formation (data not shown).

#### *The product of protein C activation by the protein C activator*

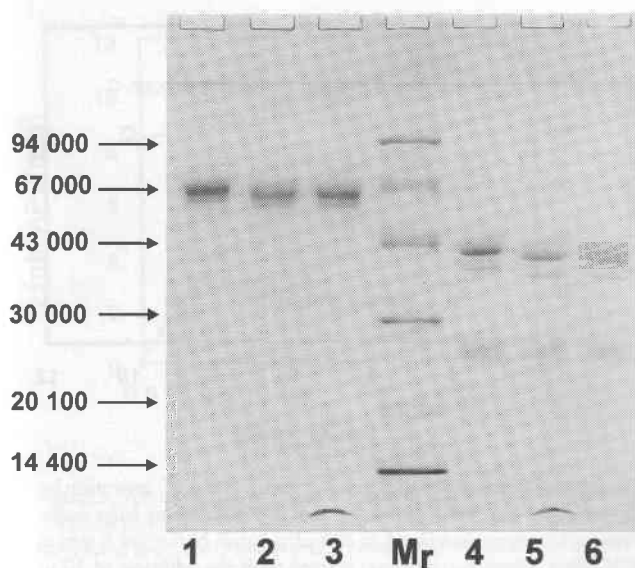
Human protein C is a protein with a molecular weight of 62 000 that consists of a heavy chain ( $M_r$  41 000)



**Figure 1.** SDS-PAGE analysis of the protein C activator from the venom of *Agkistrodon halys halys*. Crude *Agkistrodon halys halys* venom and purified activator were subjected to SDS-PAGE on 10% slabgels (6% stacking gel) according to Laemmli<sup>25</sup> under non-reducing conditions. (A): Gel stained with Coomassie Brilliant Blue R250. Lane 1, 13  $\mu$ g crude venom; Lane 2, 4  $\mu$ g purified activator; Lane 3, molecular weight standards. (B): After electrophoresis a separate part of the gel containing a lane with 4  $\mu$ g purified activator was subjected to electrophoretic transfer of the protein on to nitrocellulose and visualization of the protein C activator with protein C and S2366 as described in Materials and methods.

linked via a disulphide bridge to a light chain ( $M_r$  21 000). Activation of human protein C results from a specific cleavage in the heavy chain of the molecule.<sup>2,13,17</sup> In the case of protein C activation by its physiological activator, thrombin this cleavage has been identified to result in the removal of a dodecapeptide of  $M_r$  = 1 400 from the aminotermus of the heavy chain.<sup>2</sup>

Figure 2 shows that the protein C activator purified from *Agkistrodon halys halys* venom probably activates human protein C via the same pathway. Activation of protein C by our activator was accompanied by the cleavage of a small polypeptide from the heavy chain of protein C as indicated by the small increase in electrophoretic mobility of the heavy chain of protein C after complete activation. For comparison we have also electrophoresed the product of protein C activation by the activator from *Agkistrodon contortrix*



**Figure 2.** SDS-PAGE of protein C activated by the activators from the venoms of *Agkistrodon halys halys* and *Agkistrodon contortrix contortrix*. Human protein C (16 µg) was activated in 100 µl of a reaction buffer containing 50 mM Tris/HCl (pH 7.9 at room temperature), 0.5 mM EDTA and 0.08 µg of the purified activator from *Agkistrodon halys halys* or 0.12 µg Protac (partially purified activator from *Agkistrodon contortrix contortrix*). After complete reaction 20 µl aliquots were taken and subjected to SDS-PAGE both under non-reduced (lanes 1–3) and under reduced (lanes 4–6) conditions. A lane with molecular weight standards (identified as M<sub>r</sub>) was included on the gel. After electrophoresis the gel was stained with Coomassie Brilliant Blue R250. Lanes 1 and 4, protein C, Lanes 2 and 5, protein C activated with activator purified from the venom of *Agkistrodon halys halys*; Lane 3 and 6, protein C activated with Protac, the activator from *Agkistrodon contortrix contortrix*.

*contortrix*, which appeared to be indistinguishable from the APC generated by the activator from *Agkistrodon halys halys*. It is, therefore, highly probable that activation of protein C by the activators from both *Agkistrodon* species is due to removal of the same dodecapeptide that is also removed by thrombin.<sup>2</sup>

#### Chromogenic substrate conversion by the protein C activator

The purified protein C activator from the venom of *Agkistrodon halys halys* possesses amidolytic activity towards a number of commercially available synthetic peptide substrates (Table 2). High activities were observed on the kallikrein substrate S2302 and on the thrombin substrate S2238, whereas other substrates tested were converted at considerably lower rates. In Table 2 we have also included the rates of substrate conversion by the crude venom. From the fact that there was a considerable difference between the substrate specificities of the purified activator and the crude venom it can be concluded that the crude venom

**Table 2.** Amidolytic activities of crude *Agkistrodon halys halys* venom and of the purified protein C activator

Substrate	Rate of substrate hydrolysis	
	Crude venom (µM pNA/min/mg)	Purified activator (µM pNA/min/mg)
S2238	0.371	0.703
Chromozym TH	0.346	0.062
S2222	0.036	0.029
S2337	0.051	0.009
S2765	0.039	0.011
S2366	0.129	0.027
S2302	4.024	1.290
S2288	0.271	0.048
S2251	0.286	0.043

Amidolytic activities were determined at 37°C in 1 ml cuvettes (1 cm pathlength) on an Aminco DW2-C spectrophotometer set in the dual wavelength mode at 405 minus 500 nm. Final reaction conditions were: 50 mM Tris/HCl (pH 7.9 at room temp), 175 mM NaCl, 20 mM EDTA, 0.5 mg/ml ovalbumin, 192 µM chromogenic substrate and appropriate amounts of crude venom or purified activator. From the  $\Delta A_{405-500}/\text{min}$  the rate of *p*-nitroaniline formation was calculated using a molar extinction coefficient of 9 900 cm<sup>-1</sup>.<sup>29</sup>

from *Agkistrodon halys halys* contains other enzymes with amidolytic activity. Since the crude venom contains less than 0.2% protein C activator (as can be concluded from the 533-fold purification of the activator, Table 1) it can be calculated that the protein C activator has a negligible contribution to the amidolytic activity of the crude venom.

#### The effect of protease inhibitors on the activity of the protein C activator

Table 3 summarizes the effects of inhibitors of different classes of proteolytic enzymes on the activity of the protein C activator from *Agkistrodon halys halys*. Inhibitors of metallo- and thiol proteases did not affect the amidolytic activity of the protein C activator as determined with S2302. Considerable inhibition was observed when the protein C activator was incubated with the serine protease inhibitors benzamidin, soybean trypsin inhibitor, PPACK and *p*-NPGB. The virtually complete inhibition by pNPGB and PPACK suggests that the protein C activator from *Agkistrodon halys halys* is a serine protease in which both histidine and serine residues are involved in the catalytic mechanism.

#### Effect of CaCl<sub>2</sub> and NaCl on protein C activation

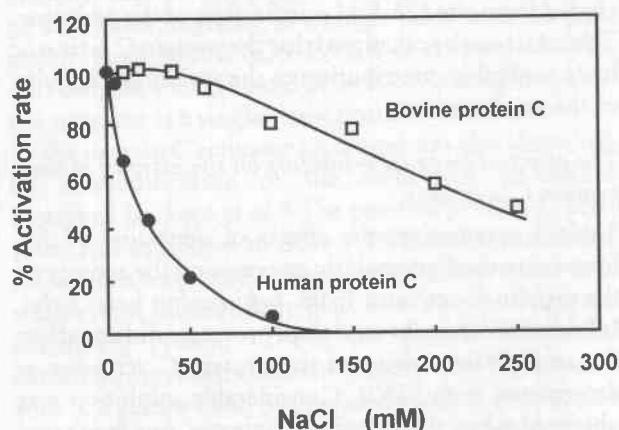
During our studies it became clear that the activator isolated from the venom of *Agkistrodon halys halys* activated both bovine and human protein C. In this respect it resembles the protein C activator from the venom of *Agkistrodon contortrix contortrix*. Our pro-



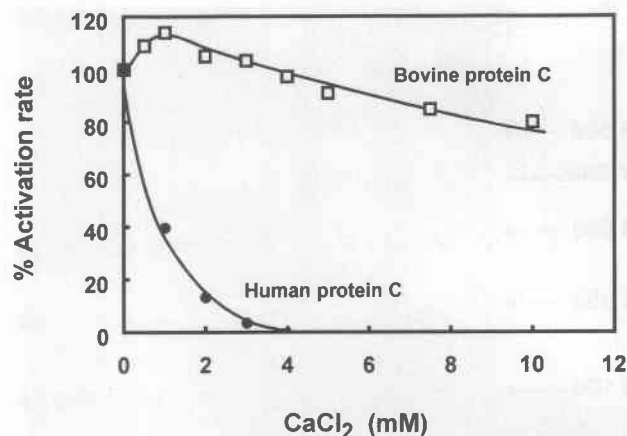
**Table 3.** Inhibition of the purified *Agkistrodon halys halys* activator by protease inhibitors

Inhibitor		% activity
none		100
<i>o</i> -phenantroline	(4 mM)	94
EDTA	(10 mM)	107
2-iodoacetamide	(1 mM)	99
<i>n</i> -ethylmaleimide	(1 mM)	97
<i>p</i> -amidino PMSF	(100 $\mu$ M)	98
antithrombin III	(60 nM)	117
benzamidine	(10 mM)	48
soybean trypsin inhibitor	(10 mg/ml)	66
<i>p</i> -NPGb	(300 $\mu$ M)	6
PPACK	(50 $\mu$ M)	4

0.77  $\mu$ g Protein C activator purified from *Agkistrodon halys halys* venom was incubated for 1 h at 37°C in 450  $\mu$ l buffer containing 50 mM Tris/HCl (pH 7.9 at room temperature), 175 mM NaCl, 0.5 mg/ml ovalbumin and inhibitor as indicated. The residual amidolytic activity was determined after 1 h by adding 50  $\mu$ l 3.84 mM S2302 in water. The reaction with chromogenic substrate was allowed to proceed (usually between 5 and 25 min) until sufficient *p*-nitroaniline was formed for accurate measurement of the absorbance at 405–500 nm after which 300  $\mu$ l 1 M citric acid was added to stop *p*-nitroaniline formation. From the measured absorbance the amidolytic activity was calculated as  $\Delta A_{405-500}$  nm/min. The percentage remaining activity was obtained by taking the activity measured in the absence of inhibitors as 100%.

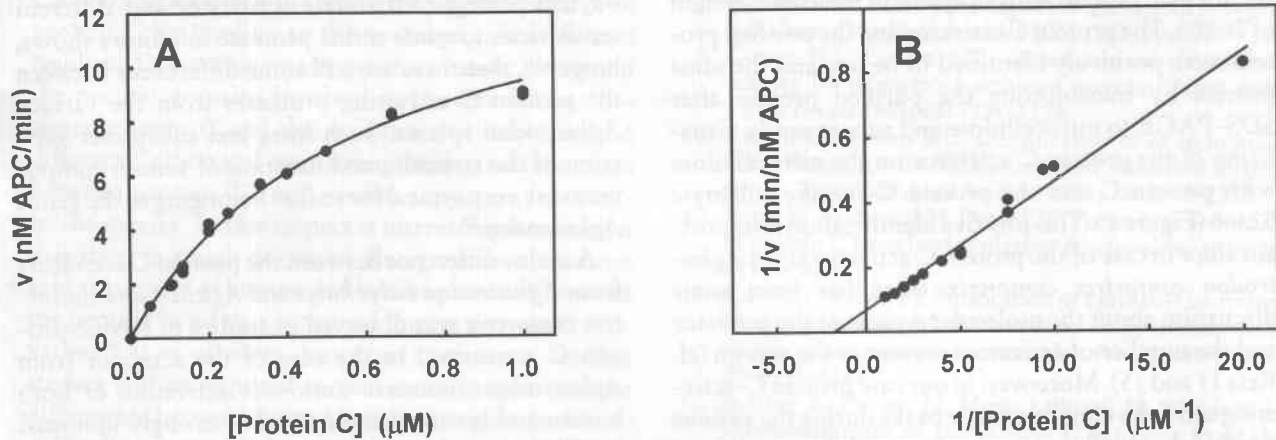


**Figure 3.** Effect of NaCl on the rate of protein C activation by the purified activator from *Agkistrodon halys halys* venom. Protein C was preincubated in 240  $\mu$ l reaction buffer for 5 min at 37°C after which reaction was started with the addition of 10  $\mu$ l buffer containing purified *Agkistrodon halys halys* activator. Final concentrations of reactants were: 50 mM Tris/HCl (pH 7.9 at room temperature), NaCl as indicated in the figure, 5 mM EDTA, 0.5 mg/ml ovalbumin, 130 nM human protein C and 25 ng purified activator (●—●) or 150 nM bovine protein C and 5 ng activator (□—□). After 1, 3 and 5 min 25  $\mu$ l aliquots were removed from the reaction mixture and assayed for activated protein C using the chromogenic substrate S2366 as described under Methods. From the amounts of activated protein C thus determined the initial rate of protein C activation was calculated. The rate of protein C activation determined in the absence of NaCl was taken as 100% (5.9 nM human APC formed per min and 5.4 nM bovine APC formed per min).



**Figure 4.** The effect of  $\text{CaCl}_2$  on the rate of protein C activation by the activator purified from the venom of *Agkistrodon halys halys*. Protein C was preincubated in 240  $\mu$ l reaction buffer for 5 min at 37°C after which reaction was started with the addition of 10  $\mu$ l buffer containing purified *Agkistrodon halys halys* activator. Final concentrations of reactants were: 50 mM Tris/HCl (pH 7.9 at room temperature), 10 mM NaCl, 0.5 mM EDTA, 0.5 mg/ml ovalbumin, 130 nM human protein C with 25 ng activator (●—●) or 150 nM bovine protein C with 4 ng activator (□—□) and amounts of  $\text{CaCl}_2$  in excess over EDTA to obtain the concentrations indicated in the figure. After 1, 3 and 5 min 25  $\mu$ l aliquots were removed from the reaction mixture and assayed for activated protein C using the chromogenic substrate S2366 as described under Methods. From the amounts of activated protein C present the initial (steady-state) rate of protein C activation was calculated and expressed as percentage of the rate determined in the absence of added  $\text{CaCl}_2$  (5.7 nM human APC formed per min and 4.2 nM bovine APC formed per min).

tein C activator differs, however, in one important aspect from the latter activator. Activation of both bovine and human protein C by the activator from *Agkistrodon contortrix contortrix* is strongly inhibited by  $\text{Ca}^{2+}$  ions<sup>14,15</sup> and (at least for human protein C) by NaCl.<sup>15</sup> When we tested protein C activation with Protac (the activator from *Agkistrodon contortrix contortrix* venom) under our experimental conditions, both bovine and human protein C activation by Protac were strongly inhibited by  $\text{CaCl}_2$  ( $K_{i \text{ app}} < 1$  mM) and by NaCl ( $K_{i \text{ app}} \approx 50$  mM) (data not shown). However,  $\text{Ca}^{2+}$  ions and increasing ionic strength had different effects on the activation of bovine and human protein C by the *Agkistrodon halys halys* protein C activator. Figure 3 shows that activation of human protein C by the activator from *Agkistrodon halys halys* was strongly inhibited at increasing NaCl concentrations ( $K_{i \text{ app}} = 20$  mM) whereas the activation of bovine protein C was inhibited at much higher NaCl concentrations ( $K_{i \text{ app}} = 220$  mM). A similar difference between bovine and human protein C activation was also observed when the effect of  $\text{Ca}^{2+}$  ions was studied (Figure 4). Activation of human protein C was already



**Figure 5.** Kinetic analysis of human protein C activation by the activator purified from the venom of *Agkistrodon halys halys*. Varying amounts of human protein C were incubated for 5 min at 37°C in 140 μl buffer after which reaction was started by addition of 10 μl purified *Agkistrodon halys halys* activator. Final reaction conditions were: 50 mM Tris/HCl (pH 7.9 at room temperature), 0.5 mM EDTA, 1 mg/ml ovalbumin, 7.7 ng purified activator and amounts of protein C as indicated in the figure. The initial rate of protein C activation was determined as described in the legend to Figure 3. (A) rate of protein C activation as a function of the protein C concentration. (B) Lineweaver-Burk plot of the same data. Further details are given under Materials and methods.

inhibited at low  $\text{Ca}^{2+}$  concentrations ( $K_{i \text{ app}} = 0.8 \text{ mM}$ ), whereas bovine protein C activation was even slightly stimulated at this calcium concentration. At much higher  $\text{Ca}^{2+}$  concentrations there was some inhibition of bovine protein C activation by the activator from *Agkistrodon halys halys*. The  $K_{i \text{ app}}$  for inhibition by  $\text{Ca}^{2+}$  ions was, however, much higher than 10 mM.

#### Kinetic parameters of protein C activation by the protein C activator

To assess the efficiency by which the purified *Agkistrodon halys halys* enzyme activated human protein C we determined the kinetic parameters of protein C activation by measuring initial rates of activation at varying protein C concentrations. The reaction appeared to be saturable with respect to protein C (Figure 5A) and obeyed Michaelis-Menten kinetics as evidenced by the straight Lineweaver-Burk plot (Figure 5B). The latter plot yields a  $V_{\text{max}}$  of 14.2 nM protein C activated per min and  $K_m$  for human protein C of 0.52 μM. From the concentration of activator present in this experiment (0.051 μg/ml) and the molecular weight of the activator (36 000) it can be calculated that the  $k_{\text{cat}}$  is 0.17 s<sup>-1</sup> and  $k_{\text{cat}}/K_m = 3.3 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$ . These kinetic parameters were determined in a reaction medium that did not contain NaCl. It was not possible to obtain kinetic parameters at high NaCl concentrations since we were not able to saturate the activator with human protein C ( $K_m > 2 \text{ μM}$ ) and from the observed rate of APC formation the second-order rate constant ( $k_{\text{cat}}/K_m$ ) was calculated to be  $4.3 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$  at 50 mM NaCl.

The activation of bovine protein C has more favour-

able kinetic parameters. At 50 mM NaCl a  $V_{\text{max}}$  of 10.4 nM APC/min and a  $K_m$  of 0.056 μM were obtained at 0.026 μg/ml activator which yields a  $k_{\text{cat}}$  of 0.24 s<sup>-1</sup> and a  $k_{\text{cat}}/K_m$  of  $4.3 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$ . The latter value indicates that at 50 mM NaCl bovine protein C is activated with a 100-fold higher catalytic efficiency than human protein C. The kinetic parameters obtained in the experiments discussed above are summarized in Table 4.

#### Discussion

In the present paper we have described the purification and the characterization of a protein C activator from the venom of *Agkistrodon halys halys*. The activator is a

**Table 4.** Kinetic parameters of protein C activation by the activator purified from *Agkistrodon halys halys* venom

Substrate	$k_{\text{cat}}$ (s <sup>-1</sup> ± SE)	$K_m$ (μM ± SE)	$k_{\text{cat}}/K_m$ (M <sup>-1</sup> s <sup>-1</sup> ± SE)
Human protein C			
I = 0.05	0.17 ± 0.007	0.52 ± 0.04	$4.3(\pm 0.4) \times 10^5$
I = 0.15	ND	ND	$3.4(\pm 0.3) \times 10^4$
Bovine protein C			
I = 0.10	0.24 ± 0.006	0.056 ± 0.004	$4.4(\pm 0.4) \times 10^6$

Kinetic parameters of protein C activation were obtained at 37°C as described under Materials and methods and under the legend to Figure 5. In the case of human protein C activation at I = 0.15 rates of APC formation were linear with respect to protein C concentration and from the observed reaction rates the second-order rate constant, which equals  $k_{\text{cat}}/K_m$ , was calculated. Further details are given in the text.

single chain protein with an apparent molecular weight of 36 000. The protein C activator and the purified protein were positively identified to be one and the same protein by transblotting the purified protein after SDS-PAGE to nitrocellulose and subsequently visualizing of the protein C activator on the nitrocellulose with protein C and the protein C-specific substrate S2366 (Figure 1). This positive identification is important since in case of the protein C activator from *Agkistrodon contortrix contortrix* there has been some discussion about the molecular weight of the activator and the number of activators present in the venom (cf. Refs 11 and 15). Moreover, in our case protein C-activating activities eluted in single peaks during the various steps of the purification procedure and thus we did not find evidence that *Agkistrodon halys halys* venom might contain more than one protein C activator.

The protein C activator from *Agkistrodon halys halys* hydrolysed several tripeptide-*p*-nitroanilide substrates and its activity was readily inhibited by *p*-NPGB and PPACK which identifies the activator as a serine protease. The activator converted both human and bovine protein C into APC by removal of a small polypeptide fragment from the heavy chain of protein C. It is very likely that protein C activation by the venom activators from different *Agkistrodon* species is the result of cleavage of the same peptide bond in protein C that is also cleaved by thrombin<sup>2</sup> yielding products with the same functional activity.

Whereas the rate of hydrolysis of tripeptide-*p*-nitroanilide substrates by the activator was not affected by variation of the  $\text{Ca}^{2+}$  and NaCl concentrations in the reaction medium there was considerable inhibition of human protein C activation in activation mixtures which contained  $\text{Ca}^{2+}$  ions or high NaCl concentrations. Fifty per cent inhibition of human protein C activation by the venom activator was observed at 0.8 mM  $\text{CaCl}_2$  and 20 mM NaCl, respectively. Kinetic studies at low ionic strength ( $I = 0.05$ ) in the absence of  $\text{Ca}^{2+}$  ions revealed an apparent  $K_m$  for human protein C of 0.52  $\mu\text{M}$  and a  $k_{\text{cat}}$  of 0.17  $\text{s}^{-1}$  from which a catalytic efficiency ( $k_{\text{cat}}/K_m$ ) of  $3.3 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$  can be calculated. Increasing the ionic strength to 0.1 caused a ten-fold decrease of  $k_{\text{cat}}/K_m$  ( $4.3 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$ ) which is at least partially due to an effect of ionic strength on the  $K_m$  for protein C which becomes  $> 2 \mu\text{M}$ .

The properties discussed thus far indicate that the protein C activator from *Agkistrodon halys halys* is remarkably similar to the protein C activators from *Agkistrodon contortrix contortrix*<sup>13-15</sup> and *Agkistrodon bilineatus*<sup>17</sup> with respect to molecular weight, catalytic efficiencies of human protein C activation and the inhibitory effects of  $\text{Ca}^{2+}$  ions and high NaCl concentrations. Differences between the amidolytic activities

on tripeptide-*p*-nitroanilide substrates and different sensitivities towards serine protease inhibitors shows, however, that there are still some differences between the protein C-activating proteases from the various *Agkistrodon* species. Such intra and subspecies variation of the structure and function of venom components are very typical for snakes belonging to the genus *Agkistrodon*.<sup>27</sup>

A major difference between the protein C activators from *Agkistrodon halys halys* and *Agkistrodon contortrix contortrix* was observed in studies of bovine protein C activation. In the case of the activator from *Agkistrodon contortrix contortrix* activation of both human and bovine protein C were strongly inhibited by  $\text{Ca}^{2+}$  ions and by high NaCl concentrations under our experimental conditions. Human protein C activation by the activator from *Agkistrodon halys halys* was also very sensitive to the presence of  $\text{Ca}^{2+}$  ions ( $K_i = 0.8 \text{ mM}$ ) and to the NaCl concentration ( $K_i = 20 \text{ mM}$ ). However, with bovine protein C much higher  $\text{CaCl}_2$  and NaCl concentrations are required in order to observe inhibition (cf. Figures 3 and 4). These observations indicate that there are considerable differences between human and bovine protein C on one hand and between the venom protein C activators on the other hand.

Orthner *et al.*<sup>15</sup> advanced the hypothesis that  $\text{Ca}^{2+}$  ions and NaCl change the conformation of protein C in such a way that it becomes a less favourable substrate for the protein C activator. Considering the experiments reported in the present paper and pursuing the proposal of Orthner and coworkers results in a complex picture with different conformations for human and bovine protein C at high  $\text{Ca}^{2+}$  and NaCl that are recognized by one activator (*Agkistrodon halys halys*) and not by the other activator (*Agkistrodon contortrix contortrix*). It is, however, also possible that other effects contribute to the observed calcium and ionic strength dependence of venom-catalysed protein C activation. Strong effects of ionic strength on chemical reactions are often taken to implicate that ionic (electrostatic) forces play an essential role in the interactions between the reacting substances. In the case of protein C activation by the venom activators this would mean that ionic interactions between protein C and the venom activator may have an important contribution to the formation of the enzyme-substrate complex and the subsequent proteolysis. Considering the properties of protein C and the venom activators from the various *Agkistrodon* species this will likely be an ionic interaction between a negatively charged domain on protein C and a positively charged protein domain on the venom activator. Such an interaction will be prevented both at increased ionic strength and also by



Ca<sup>2+</sup> ions, since this will reduce the negative charge of protein C by binding to the  $\gamma$ -carboxyglutamic acid residues. Depending on the net electrostatic charges of the protein domains involved in the interactions between protein C and the activator this may result in different Ca<sup>2+</sup> ion and ionic strength effects on reactions between protein C's and venom activators from different species. In this respect it is interesting to mention that there is a high degree of divergence in the amino acid sequences of human and bovine protein C around the peptide bond that is cleaved during protein C activation.<sup>28</sup> It is obvious, however, that more detailed studies will be required to gain more insight in these differences between human and bovine protein C.

Finally we would like to emphasize that it is of interest to study the functional properties of the protein C activators present in the venoms of other *Agkistrodon* species. Our experiments indicate that these protein C activators may exhibit a number of important functional differences. It is, therefore, possible that other *Agkistrodon* species contain a protein C activator whose activity on human protein C is not inhibited by Ca<sup>2+</sup> ions and high NaCl concentrations. Such an activator will be the preferred enzyme in a diagnostic test for the quantitation of plasma protein C.

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